Molecular Properties of the Erythromycin Resistance Plasmid pPV141 from *Staphylococcus chromogenes*

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The 2.3-kb erythromycin resistance (Em^R) plasmid pPV141 of *Staphylococcus chromogenes* 3688 was isolated and characterized. Nucleotide sequence analysis identified ORF1 and ORF2 separated by a 445-bp spacing, encoding a 158-residue replication protein (Rep141) and a 244-residue erythromycin resistance protein (Erm, rRNA adenine N-6-methyltransferase), respectively. Structural analysis and Southern hybridization showed that the *rep* and *ermM* genes in pPV141 shared homology with other known Em^R plasmids. Based on sequence analysis, pPV141 was classified as a unique member of the pSN2 family of Em^R plasmids.

Plasmid-borne resistance to a variety of antibiotics in human isolates of coagulase negative staphylococci (*Staphylococcus epidermidis, S. simulans*) is an established fact and several resistance plasmids from these organisms have been extensively studied (Lyon and Skurray, 1987; DeGuglielmo *et al.,* 1991; Barcs and Janosi, 1992). Veterinary strains of *S. epidermidis, S. simulans, S. hyicus, and S. chromogenes* (formerly *S. hyicus* ssp. *chromogenes,* Hajek *et al.,* 1986) are commonly associated with cattle and swine and, similarly to human isolates, may be involved as opportunistic pathogens in the pathology of epidermitis, otitis, and mastitis (Holmberg, 1973; Devriese, 1979; Kloos *et al.,* 1981). Although the occurrence of plasmids in veterinary strains of *S. hyicus* was reported earlier (Kloos *et al.,* 1981), they remained largely uncharacterized until the identification of several antibiotic resistance plasmids by Noble *et al.* (1988). Since that time, plasmids of *S. hyicus* encoding resistance to chloramphenicol (Schwarz *et al.,* 1989), tetracycline (Schwarz and Blobel, 1990a; Schwarz *et al.,* 1992), streptomycin (Schwarz and Blobel, 1990b; Schwarz and Noble, 1994), and erythromycin (Schwarz *et al.,* 1990; Wegener and Schwarz, 1993) have been studied in detail. On the other hand, the plasmid biology of *S. chromogenes, S. epidermidis,* and *S. simulans* from veterinary sources has remained largely unexplored.

The molecular characterization of antibiotic resistance plasmids in veterinary strains of coagulase negative staphylococci is significant from two perspectives. First, it may yield information on the degree of relatedness among resistance plasmids in these microorganisms and between resistance plasmids of human- and animal-hosted strains of staphylococci. Second, information on genetic markers of these plasmids may be of interest in the development of vectors with readily selectable genetic markers that may find application in the genetic manipulation of related and unrelated Gram-positive microbes.

We have previously reported the successful use of the *erm* gene of pPV141, a 2.3-kb Em^R plasmid present in *S. chromogenes* 3688, as a reporter gene in vector constructs with shuttle capacity (Solaiman and Somkuti, 1993, 1995).

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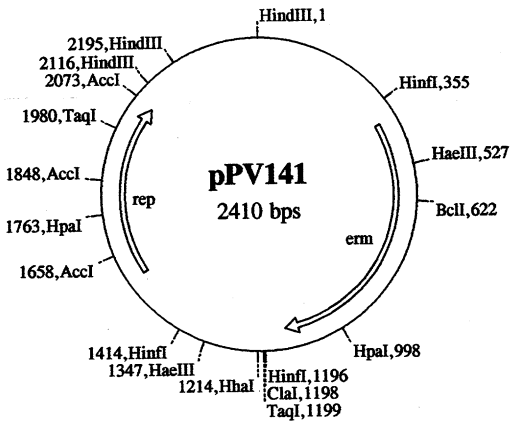


FIG. 1. Restriction cleavage map of pPV141 from *S. chromogenes* 3688. Arrows designate ORF1 (*rep*) and ORF2 (*erm*).

In this paper, we report the detailed structural analysis of the *rep* and *erm* regions and the complete nucleotide sequence of pPV141 from *S. chromogenes* 3688. The properties of pPV141 are also compared with those of other Em^R plasmids that have been described in the literature.

MATERIALS AND METHODS

Microbial Strains and Maintenance

The coagulase negative *S. chromogenes* 3688 (mastitis, cow) with Em^R phenotype was speciated with 93%+ accuracy by the API STAPH Track kit (API Laboratory Products, Ltd., St. Laurent, Quebec) and supplied by the Animal Disease Diagnostic Laboratory, Purdue University School of Veterinary Medicine (West Lafayette, IN). Control cultures with Em^R plasmids included *S. epidermidis* (pNE131, a gift from J. T. Parisi) and *S. aureus* (pE194, a gift from B. Weisblum). The staphylococci were grown at 37°C for 24 h in tryptic soy broth (TSB, Difco Laboratories, Detroit, MI), supplemented with erythromycin at 15 μg/ml, and then stored at 4°C between weekly transfers.

Plasmid Profiles and Curing

Plasmids were isolated by a procedure previously described (Somkuti and Steinberg, 1986) with the inclusion of lysostaphin (Sigma Chemical Co., St. Louis, MO) at 50 μg/ml in the digestion mixture. Plasmid composition was determined by agarose gel electrophoresis (AGE) in 0.7% agarose (FMC Corporation, Rockland, ME) in a Tris/borate/EDTA buffer system (0.089 M Tris base, 0.089 M boric acid, 0.002 M EDTA, pH 8.3), at 100 V for 4 h.

Cultures were cured of Em^R phenotype by exposure to ethidium bromide at 10 μM for 16 h. After serial dilution, samples were plated on TSB with 1.5% agar. Colonies were toothpicked into 96-well microtiter plates (Vangard International, Inc., Neptune, NJ), with 200 μl TSB per well. After 16 h incubation, cultures were replica-plated into TSB with 15 μg/ml erythromycin. Plates were scored for turbidity after 24 h. Erythromycin-sensitive clones were counterselected from the original set of plates and screened for plasmids.

DNA Analysis and Manipulations

The putative Em^R plasmid of *S. chromogenes* 6388 was further purified by CsCl density gradient centrifugation (Stougaard and Molin, 1981)

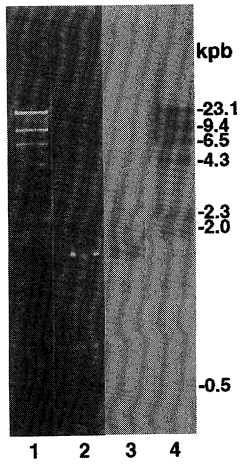


FIG. 2. Agarose gel electrophoretic (lane 2) and Southern hybridization (lane 3) patterns of *Taq*I-digested pPV141 with a biotinylated *Taq/Hin*dIII fragment of pNE131 as the probe; lanes 1 and 4 show a *Hin*dIII-digested λ DNA control. The use of a biotinylated *Taq*I fragment from pE194 as probe yielded identical results.

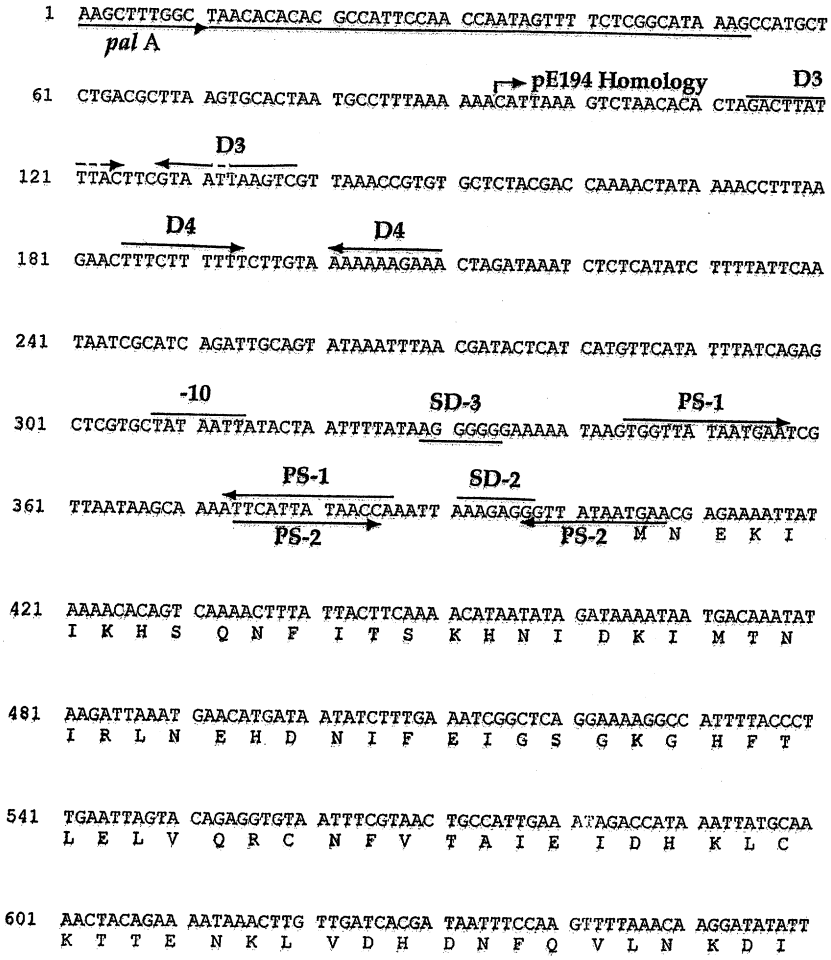


FIG. 3. Complete nucleotide sequence of pPV141 numbered from the *Hin*dIII site shown as the 0 coordinate in Fig. 1. Homologies with pE194 (▶) and pSN2 (▶▶) are indicated. Open reading frames (ORF1 and ORF2) with their potential Shine–Dalgarno (SD) sequences and the amino acid sequences of putative polypeptides are indicated. Converging arrows indicate complementary base-paring sequences (PS) and dyad symmetries (D). The sequence of pPV141 has been deposited in GenBank under Accession No. U82607.

or by Elutip-d treatment (Schleicher & Schuell, Inc., Keene, NH) and mapped by single and coupled digestions with an array of restriction endonucleases (BRL Life Technologies, Inc., Gaithersburg, MD) under conditions recommended by the manufacturer. DNA fragments were analyzed in 1.2% agarose gels under conditions described above.

To approximate the position of the *erm* region, restriction endonuclease fragments were spliced into pBR322 with T4 DNA ligase using the *Cla*I and *Hin*dIII insertion sites under conditions recommended by the vendor (United States Biochemical, Cleveland, OH). Ligation mixtures were used for direct transformation of freshly prepared competent cells (Sambrook *et al.*, 1989) of *Escherichia coli* DB11, a highly Em-sensitive variant of *E. coli* K-12 (provided by J. Davies). Em^R clones were scored after growth on Luria–Bertani (LB) agar plates (1% tryptone, 0.5% each of yeast extract and NaCl, and 1.5% agar) supplemented with ampicillin (100 μg/ml) and erythromycin (25 μg/ml).

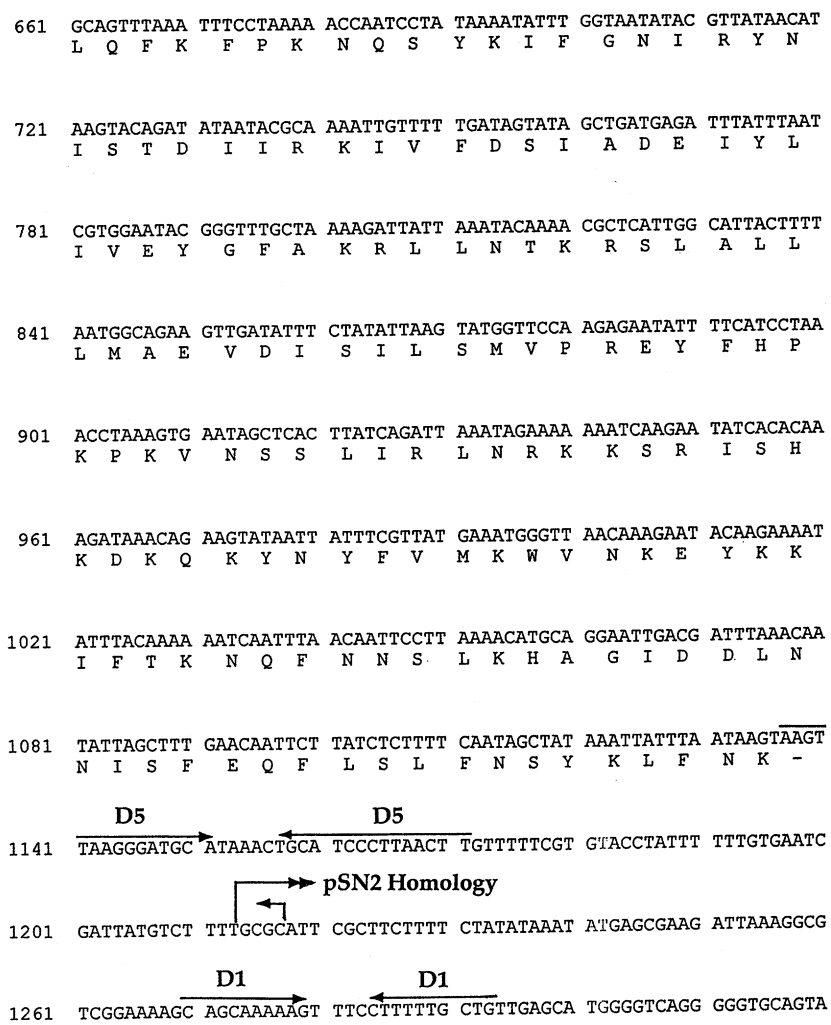
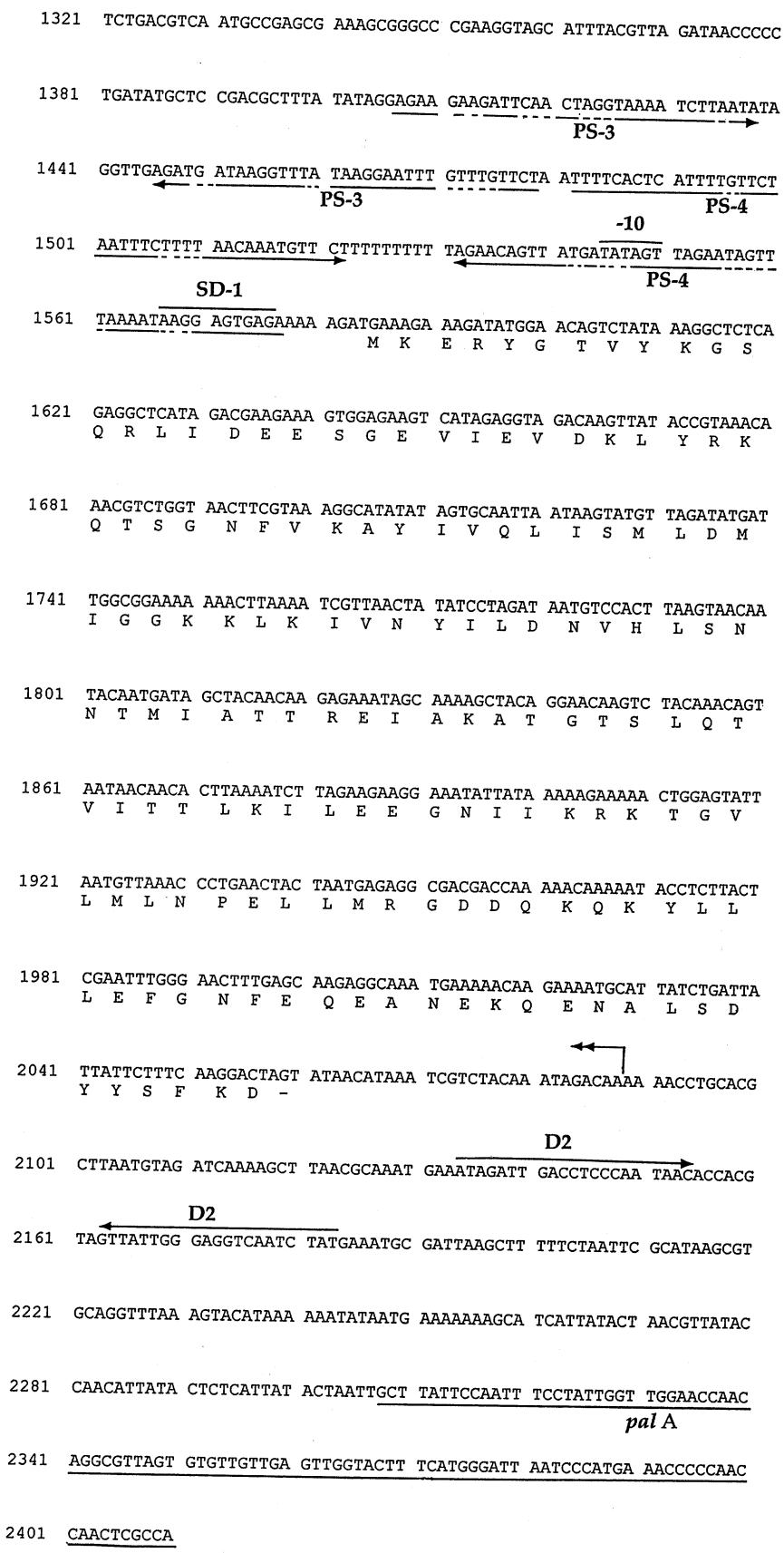


FIG. 3—*Continued*

Biotinylated probes were prepared from a ca. 1.4-kb *Taq*I fragment of pE194 from *S. aureus* (Horinouchi and Weisblum, 1982) and a ca. 1.6-kb *Taq*I/*Hin*dIII fragment of pNE131 from *S. epidermidis* (Lampson and Parisi, 1986a), each corresponding to the *erm* region of these plasmids, according to the method of Leary *et al.* (1983). Southern blots with the putative Em^R plasmid (pPV141) of *S. chromogenes* 6388 digested with *Taq*I were prepared in an Automated Southern Blot System (Oncor, Inc., Gaithersburg, MD), according to the manufacturer's recommendations, at 45% formamide concentration.

Fragments from restriction endonuclease digestions of pPV141 were spliced into appropriate polylinker cloning sites on pUC19. Ligation products were used to transform competent cells of *E. coli* DH5α (BRL Technologies) and recombinant (white) clones were selected on LB agar (see above) supplemented with 100 μg/ml ampicillin and 50 μg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Recombinant plasmids were isolated and purified and DNA sequencing was carried out in triplicate by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) in an ALF DNA Sequencer unit (Phar-


1321 TCTGACGTCA ATGCCGAGCG AAAGCGGGCC CGAAGGTAGC ATTTACGTTA GATAACCCCC
1381 TGATATGCTC CGACGCTTTA TATAGGAGAA GAAGATTCAA CTAGGTAAAA TCTTAATATA
PS-3
1441 GGTTGAGATG ATAAGGTTTA TAAGGAATTT GTTTGTTCTA ATTTTCACTC ATTTTGTTCT
PS-3
PS-4
-10
1501 AATTTCTTTT AACAAATGTT CTTTTTTTTT TAGAACAGTT ATGATATAGT TAGAATAGTT
PS-4
SD-1
1561 TAAAATAAGG AGTGAGAAAA AGATGAAAGA AAGATATGGA ACAGTCTATA AAGGCTCTCA
M K E R Y G T V Y K G S
1621 GAGGCTCATA GACGAAGAAA GTGGAGAAGT CATAGAGGTA GACAAGTTAT ACCGTAAACA
Q R L I D E E S G E V I E V D K L Y R K
1681 AACGTCTGGT AACTTCGTAA AGGCATATAT AGTGCAATTA ATAAGTATGT TAGATATGAT
Q T S G N F V K A Y I V Q L I S M L D M
1741 TGGCGGAAAA AAACTTAAAA TCGTTAACTA TATCCTAGAT AATGTCCACT TAAGTAACAA
I G G K K L K I V N Y I L D N V H L S N
1801 TACAATGATA GCTACAACAA GAGAAATAGC AAAAGCTACA GGAACAAGTC TACAAACAGT
N T M I A T T R E I A K A T G T S L Q T
1861 AATAACAACA CTTAAAATCT TAGAAGAAGG AAATATTATA AAAAGAAAAA CTGGAGTATT
V I T T L K I L E E G N I I K R K T G V
1921 AATGTTAAAC CCTGAACTAC TAATGAGAGG CGACGACCAA AAACAAAAAT ACCTCTTACT
L M L N P E L L M R G D D Q K Q K Y L L
1981 CGAATTTGGG AACTTTGAGC AAGAGGCAAA TGAAAAACAA GAAAATGCAT TATCTGATTA
L E F G N F E Q E A N E K Q E N A L S D
2041 TTATTCTTTC AAGGACTAGT ATAACATAAA TCGTCTACAA ATAGACAAAA AACCTGCACG
Y Y S F K D -
D2
2101 CTTAATGTAG ATCAAAAGCT TAACGCAAAT GAAATAGATT GACCTCCCAA TAACACCACG
D2
2161 TAGTTATTGG GAGGTCAATC TATGAAATGC GATTAAGCTT TTTCTAATTC GCATAAGCGT
2221 GCAGGTTTAA AGTACATAAA AAATATAATG AAAAAAAGCA TCATTATACT AACGTTATAC
2281 CAACATTATA CTCTCATTAT ACTAATTGCT TATTCCAATT TCCTATTGGT TGGAACCAAC
pal A
2341 AGGCGTTAGT GTGTTGTTGA GTTGGTACTT TCATGGGATT AATCCCATGA AACCCCCAAC
2401 CAACTCGCCA

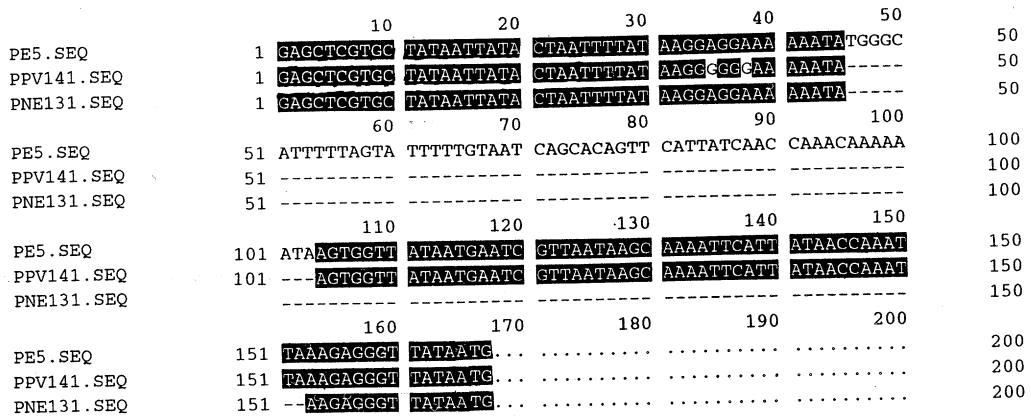


FIG. 4. Comparison of nucleotide sequences encompassing the leader mRNA and stretches 5′ to SD-2 (*erm*) in Em^R plasmids. Note the absence of the leader mRNA in pPV141 and pNE131 but the retention of a 49-bp sequence in the former which allows the formation of PS-1 or PS-2 (see Fig. 3).

macia, New Brunswick, NJ), with a T7 Autoread Sequencing kit using M13 universal and M13 reverse primers. Putative −10 and Shine–Dalgarno sequences were identified with the aid of the Clone Manager Program—Version 4 (Scientific and Educational Software, State Line, PA). Sequence comparison of pPV141 with other Em^R plasmids was done with the aid of BLASTP and BLASTX database programs (Altschul *et al.,* 1990). Multiple sequence alignments were carried out using DNASIS WINDOWS 2.1 (Hitachi Software Engineering America, San Bruno, CA).

RESULTS AND DISCUSSION

Structural Analysis of Em^R Plasmids

AGE analysis detected three plasmids (2.4, 3.5, and 40 kb) in *S. chromogenes* 3688. The molecular mass of the smaller plasmids were similar to that other coagulase-negative staphylococci surveyed by Kloos *et al.* (1981) and Noble *et al.* (1988), whereas the presence of the 40-kb plasmid maybe atypical for this species.

In curing experiments, exposure of *S. chromogenes* 3688 to ethidium bromide resulted in the loss of the Em*R* phenotype and the disappearance of the 2.4-kb (pPV141) band from the plasmid profile. The frequency of curing was 7.5×10^{-2}.

The circular restriction endonuclease map of pPV141 is shown in Fig. 1. Confirmation of pPV141 as an Em^R plasmid came from subcloning various restriction endonuclease fragments into pBR322 and using the recombinant constructs to transform competent cells of *E. coli* DB11. Transformed DB11 clones with

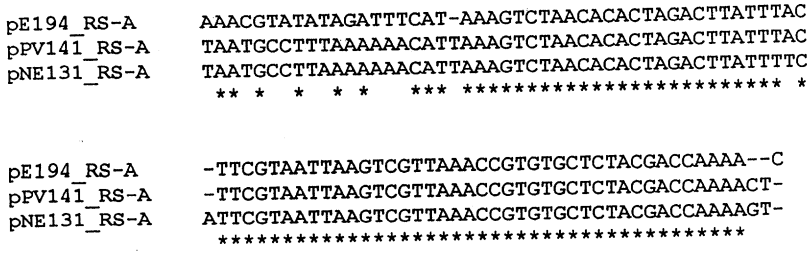


FIG. 5. Comparison of the recombination sequence RS-A in pE194, pPV141, and pNE131. Asterisks indicate positions of conserved bases.

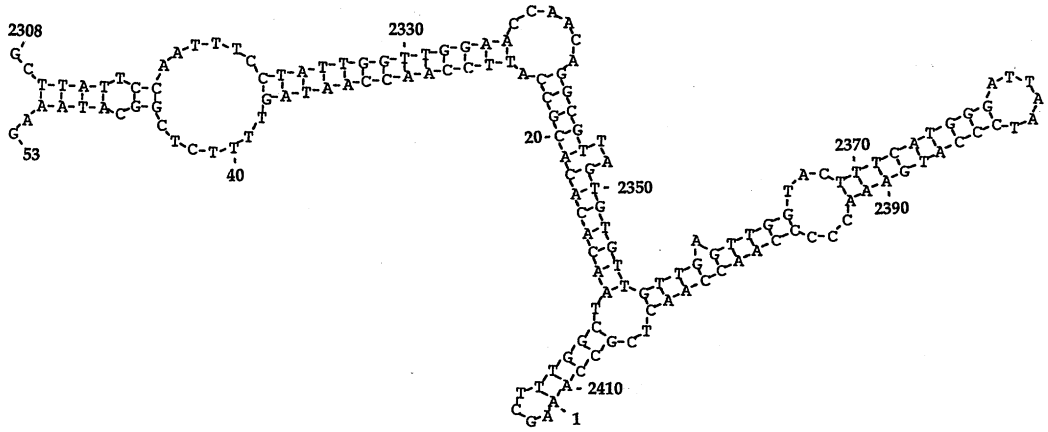


FIG. 6. Possible hairpin loop structure for the *pal*A sequence of pPV141. The free energy of formation for this structure is −88.3 kcal/mol.

erythromycin resistance up to 100 μg/ml concentration were further analyzed. The Em^R phenotype was associated with the cloning of a ca. 1.2-kb *Cla*I/*Hin*dIII (coordinates 0–1.2) fragment of pPV141. Strong signals in Southern probes of *Taq*I-digested pPV141 with biotinylated fragments (*erm*) of either pNE131 (*S. epidermidis*) or pE194 (*S. aureus*) indicated homology with these staphylococcal Em^R plasmids (Fig. 2).

Molecular Characterization of pPV141

The complete nucleotide sequence of pPV141 is shown in Fig. 3. The plasmid was 2410 bp long and had two major ORFs in the same reading frame separated by a 445-bp spacing. The larger ORF2 apparently encoded the nucleotide sequence of the 244-amino-acid *erm* (coordinates 404 to 1138) gene product which showed a high degree of homology (97%+) with other *erm* sequences found in plasmids isolated from *S. aureus* (pT48, Catchpole *et al.,* 1988; pE5, Projan *et al.,* 1987; pE194, Horinouchi and Weisblum, 1982), *S. epidermidis* (pNE131, Lampson and Parisi, 1986a), and *Bacillus subtilis* (pIM13, Monod *et al.,* 1986). To a lesser degree (75%), the protein product of *erm* in *S. chromogenes* 3688 also shared homology with the 244-amino-acid Em^R protein encoded on plasmid pGT633 in *Lactobacillus reuteri* (Tannock *et al.,* 1994). Detailed analysis of the 5′ region of ORF2 revealed the presence of two overlapping palindromic sequences (PS-1 and PS-2, Fig. 3) reminiscent of the hairpin structures involved in the posttranscriptional attenuation of *ermC* expression in pE194 (Mayford and Weisblum, 1985), pE5 (Projan *et al.,* 1987) and pT48 (Catchpole *et al.,* 1988), and *ermGT* expression in pGT633 (Tannock *et al.,* 1994), respectively. The formation of PS-1 or PS-2 in pPV141 is made possible by the unique retention of a 49-bp stretch 5′ to the Shine–Dalgarno sequence (SD-2) of the methyltransferase gene (*erm*) (Fig. 4), which is absent in both pNE131 and pIM13 (Lampson and Parisi, 1986b; Projan *et al.,* 1987). However, as in the case of pNE131 and pIM13, the lack of a potential leader mRNA coding region in pPV141 precludes the occurrence of posttranscriptional regulation of ORF2 expression (Figs. 3 and 4). This notion was supported by the results of subculturing experiments showing the constitutive expression of Em^R phenotype in pPV141-containing cells that had been repeatedly transferred (100 rounds) in the absence of the antibiotic. These results strongly suggested that ORF2 in pPV141 codes for a class *ermM* rRNA methyltransferase (Lampson and Parisi, 1986a,b). Sequence alignment studies further revealed that the ORF2 is lo-

cated in a region (coordinates 94 to 1217, Fig. 3) that is highly homologous to the pE194 *erm* region. As with the pE194 homologs of pNE131 and pIM13, the dyad symmetries D3, D4, and D5 could be found in pPV141 at nucleotide coordinates 114–138, 185–210, and 1139–1171, respectively (Fig. 3). Near the region where the pE194 homolog begins (coordinate 94), the recombination sequence RS-A similar to those found in pE194 and pNE131 could be identified. The RS-A of pPV141 exhibited a somewhat higher degree of homology with that of pNE131 than pE194 (Fig. 5). This sequence might have been involved in the recombination events that led to the formation of pPV141.

The smaller ORF1 (coordinates 1583 to 2059) apparently delineated the *rep* gene and encoded a 158-amino acid protein. The product of *rep* in pPV141 was apparently identical and shared 100% homology with replication and maintenance proteins reported for the Em^R plasmids pT48 (158/158, *S. aureus,* Catchpole *et al.,* 1988) and pE5 (158/158, *S. aureus,* Projan *et al.,* 1987). It also shared a high level of identity with *rep* gene products of Em^R plasmids pNE131 (158/162, *S. epidermidis,* Lampson and Parisi, 1986a) and pIM13 (144/146, *B. subtilis,* Projan *et al.,* 1987), as well as two small cryptic plasmids previously characterized in *S. aureus,* pSN2 (144/158, Khan and Novick, 1982) and pOX1000 (146/158, Dyke and Curnock, 1989). The *rep* gene of pPV141 is located in a region analogous to the sequence previously characterized as the pSN2-homolog in pIM13 and pNE131. One dyad symmetry, D1 (coordinates 1270 to 1293, Fig. 3) was identified in this region of pPV141 that spans from nucleotide number 1214 to 2088. The two palindromic sequences (PS-3 and PS-4, Fig. 3) located immediately upstream from the *rep* gene may be structures involved in the regulation of *rep* expression in an unknown manner. As in the case with pIM13 and pNE131 plasmids, the dyad symmetry D2 (nt 2134–2183, Fig. 3) and the minus-origin (M-O) palindromic *pal*A sequence (nt 2308–2353, Fig. 3) were also located in the region between pSN2-homolog and pE194-homolog sequences of pPV141. Computer analysis showed that a hairpin structure with $\Delta G = -88.3$ kcal/mol could be formed by the *pal*A sequence of pPV141 (Fig. 6). The high homology between the Rep sequences of pPV141, pIM13 and pNE131, and the similarity of sequence features surrounding the *rep* genes suggest that the *S. chromogenes* Em^R plasmid is a single-stranded replicon that belongs to the pSN2 group (Gruss and Ehrlich, 1989).

On the basis of the data obtained, it was reasonable to assume that pPV141 found in *S. chromogenes* 3688 shares a common evolutionary origin with other known staphylococcal Em^R plasmids.

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